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cDNA Cloning of Viroid-Induced Tomato Pathogenesis-Related Protein P23[1]

Characterization as a Vacuolar Antifungal Factor

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A 23-kD pathogenesis-related protein (P23) is induced in tomato (*Lycopersicon esculentum* Mill, cv Rutgers) plants when infected with citrus exocortis viroid. This protein is homologous to the salt-induced tomato NP24 protein (I. Rodrigo, P. Vera, R. Frank, V. Conejero [1991] Plant Mol Biol 16: 931–934). Further characterization of P23 has shown that this protein accumulates in vacuoles in association with dense inclusion bodies. In vitro assays indicated that the purified P23 protein inhibits the growth of several phytopathogenic fungi. P23-coding cDNA clones were isolated from viroid-induced and ethylene-induced libraries. Southern analysis showed that at least two genes could encode P23 or P23-related products. The accumulation of P23 protein correlated with the accumulation of its mRNA. Sequence analysis revealed significant differences in both coding and downstream untranslated regions between the cDNA sequences corresponding to the viroid-induced P23 and the salt stress-induced NP24 proteins.

Plant reaction against pathogen attack involves a number of physiological and biochemical adaptations. These include cell wall lignification, synthesis of antimicrobial low-mass compounds (phytoalexins), and the de novo expression of a number of proteins, particularly the synthesis and accumulation of a set of host-encoded PR proteins (for review, see Bowles, 1990; Dixon and Lamb, 1990). PR proteins are evolutionarily conserved in the plant kingdom and are induced by different stress situations provoked by biotic or abiotic pathogenic agents (for review, see Van Loon, 1985; Bol et al., 1990; Linthorst, 1991). Because many PR proteins display enzymic activities, such as chitinase, β-1,3-glucanase, and protease activities (Kauffmann et al., 1987; Legrand et al., 1987; Vera and Conejero, 1988), as well as other putative antimicrobial activities (Geoffroy et al., 1990), they are considered part of the defense arsenal against invading pathogens.

Systemic infection of tomato plants with CEVd results in the accumulation of up to 10 PR proteins (Granell et al., 1987). Tomato PR proteins are mainly cationic, and among them are chitinases and β-1,3-glucanases (Fischer et al., 1989; Joosten and De Witt, 1989; García-Breijo et al., 1990), as well as the sulfhydryl-protease P69 (Vera and Conejero, 1988) and the protein P1(p14) (Vera et al., 1988). We have described a 23-kD viroid-induced tomato PR protein (P23) that belongs to the thaumatin-like family and displays almost complete peptide sequence homology to the salt-induced tomato NP24 protein (Rodrigo et al., 1991). This suggests that the wide range of responses provoked by viroids in the host plant might include production of osmotins.

To characterize further the role of P23 in plant pathogenesis, we have studied the subcellular localization of this protein. In view of recent reports concerning the antifungal activity of osmotins and other thaumatin-like proteins (Vigers et al., 1991, 1992; Woloshuk et al., 1991), we also evaluated the antifungal activity of P23. In addition, several cDNA clones encoding for tomato P23 were isolated, and the complete sequence of one of them (pTCP23.1) is presented. Sequence analysis provides evidence that, unlike what peptide sequence homologies could suggest, the viroid-induced P23 and the salt-induced NP24 proteins might be encoded by different genes and induced under different stressful situations.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato plants (*Lycopersicon esculentum* Mill, cv Rutgers) were grown from seeds (Atlee Burpee Co.) in compost pots under standard greenhouse conditions. Inoculations with CEVd were performed on 12-d-old seedlings as previously described (Granell et al., 1987). Leaves showing signs of severe viroid disease were harvested under liquid N_2 25 to 30 d after inoculation and immediately used or stored frozen at −80°C.

Protein Extraction

All of the operations were carried out in an ice bath at 4°C. Plant material was homogenized in acidic extraction buffer

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Abbreviations: CEVd, citrus exocortis viroid; PR, pathogenesis related.

(84 mM citric acid, 32 mM HNa_2PO_4, 15 mM 2-mercaptoethanol [pH 2.8]), 1 mL of buffer g^{-1} of fresh tissue. The homogenate was filtered through cheesecloth and clarified by centrifugation at 20,000*g* for 20 min. The supernatant was used for protein analysis or purification and stored frozen at −80°C.

Purification of Tomato PR P23 Protein

All operations were performed at 4°C. Crude extracts from 30 g of CEVd-infected tomato leaves were adjusted to 20% (w/v) saturation with solid ammonium sulfate and centrifuged at 20,0000*g* for 20 min. The supernatant was then brought to 70% saturation with solid ammonium sulfate and recentrifuged. The resulting pellet was equilibrated in acetate buffer (50 mM sodium acetate, pH 5.5) with Sephadex G-25 (PD-10; Pharmacia) minicolumns. The eluted proteins were chromatographed on an SP-Sephadex C-25 (Pharmacia) column (1.5 × 5.5 cm), and a linear salt gradient (200 mL, 0–0.2 M NaCl in acetate buffer) was used at a flow rate of 15 mL h^{-1} to elute the retained proteins. Fractions enriched in P23 protein were applied to a fast protein liquid chromatography system (Pharmacia) using a Mono S HR 5/5 column. Proteins were eluted with a linear NaCl gradient (15 mL, 0–0.2 M in acetate buffer) at a flow rate of 1 mL min^{-1}. The protein peak corresponding to P23 was collected and stored at −20°C.

Proteins were analyzed by SDS-PAGE (14% acrylamide) as previously described (Conejero and Semancik, 1977).

Incubation of Leaf Discs with Ethylene

Leaf discs (1.4-cm diameter) were cut from fully expanded tomato leaves and incubated with 1 mM ethylene as previously described (Vera and Conejero, 1990). Discs were withdrawn from induction buffer at different times and immediately frozen with liquid N_2 and then stored at −80°C until used.

Antibodies, Immunoblots, and Immunocytochemical Techniques

Anti-P23 serum was obtained by injecting female New Zealand rabbits with purified preparations of P23 following standard procedures. For western blot immunoassay, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using electrotransfer equipment (Bio-Rad). Membranes were blocked with 2% nonfat dry milk in Tris-buffered saline and incubated with the antiserum (diluted 1:300 in blocking buffer). Antigen-antibody complexes were revealed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and 4-chloro-1-naphthol/H_2O_2 staining (Bio-Rad).

Tissue sample preparation for EM was performed as previously described (Vera et al., 1988, 1989a, 1989b). Ultrathin sections were placed on 200-mesh carbon-coated nickel grids and processed for protein A-colloidal gold immunostaining using the specific P23 antisera diluted 1:200. Controls were processed in the same way using preimmune antisera or PBS. Sections were observed in a Philips 300 electron microscope at 80 kV.

Assay of Antifungal Activity

All operations were carried out under sterile conditions. Fungi were grown in the dark at 22°C for 48 to 72 h in 3.5-cm diameter Petri dishes containing potato dextrose agar (Difco). Before the assay was conducted, the purified P23 preparation (100 μg of protein mL^{-1}) was dialyzed against 15 mM $KHPO_4$ (pH 6.0) and 20 mM NaCl (Woloshuk et al., 1991). The plant pathogenic fungi *Trichothecium roseum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum coccodes*, and *Phytophthora citrophthora* were obtained from our culture collection.

The antifungal assay with *T. roseum*, *F. oxysporum*, and *C. coccodes* was performed as follows: 2 mL of nutrient agar were poured on Petri dishes and allowed to solidify. Fungal spores were suspended at 5000 spores mL^{-1} in 100 μL of P23 solution or phosphate buffer, and the mixture was uniformly spread on an agar plate. Antifungal activity was measured for these fungi as the number of surviving colonies as a function of P23 concentration. For *P. citrophthora*, hyphae-containing agar plugs (1 mm^2) were placed on the center of agar-filled Petri dishes in the presence of 100 μL of inhibitor solution or phosphate buffer. The antifungal activity of P23 was measured as a decrease in the radial growth rate.

Nucleic Acid Purification and Hybridization Analyses

Total tomato RNA was purified according to the protocol described by Logemann et al. (1987). Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose (Sigma). For northern blot analysis, 15 μg of total RNA were electrophoresed on 1% agarose gels containing 2.2% formaldehyde and blotted onto Nytran membranes (Schleicher & Schuell). P23 probes were labeled with [α-^{32}P]dCTP (NEN-DuPont) by random priming using T7 polymerase. Hybridization conditions and washes were performed as described by Church and Gilbert (1984).

Total genomic tomato DNA was prepared by the cetyltrimethylammonium bromide precipitation procedure (Doyle and Doyle, 1990). For Southern blotting, 10 μg of DNA were digested with *Hin*dIII and *Bgl*II (Promega) and run in 0.7% agarose gels, then transferred onto Nytran membranes, and probed as indicated above.

Cloning of P23 cDNA

Poly(A)$^+$ RNA was isolated from viroid-infected tomato leaves or from tomato leaf tissue incubated for 72 h in 1 mM ethylene, and cDNA libraries were constructed in λZAP II vector (Stratagene). An ethylene-induced specific cDNA library was obtained by differential hybridization (our unpublished data), and a cDNA clone containing a partial sequence of P23 (pTE28) was isolated. The pTE28 insert was purified and used to screen both CEVd-induced and ethylene-induced tomato cDNA libraries. After more than 40,000 plaques were screened, as many as 12 positive clones were selected and converted to the pBluescript SK$^-$ form by co-infection with R408 helper phage.

Clones were sequenced on both strands using a Pharmacia T7 sequencing kit. The complete sequences were obtained by progressive nested deletions. Sequence search and analyses were performed using the FASTA, MAP, and BESTFIT rou-

tines of the University of Wisconsin's Genetics Computer Group package (Devereux et al., 1984).

RESULTS

Purification of PR P23 Protein from Viroid-Infected Tomato Leaves

Among the PR proteins that are induced in tomato plants when infected with CEVd, P23 is one of the most evident (Fig. 1, lane 1). During purification, the basic nature of P23 was confirmed because this protein was retained in cation-exchange gels such as SP-Sephadex and Mono S columns. As shown in Figure 1, a high degree of purification was achieved by following the procedure described in "Materials and Methods." Rabbit antisera were produced against the purified protein, and their monospecificity was tested by immunoblotting.

Immunocytochemical Localization of P23

To study the subcellular localization of the viroid-induced P23, tomato leaf tissue showing signs of the disease was selected. Figure 2A shows that P23 is present in the vacuole of leaf mesophyll cells in association with dense inclusion bodies. Unlike other tomato PR proteins (Vera et al., 1988, 1989a, 1989b), P23 was not detected in the leaf intercellular spaces. No significant labeling was found in ultrathin sections of CEVd-infected tomato leaves incubated with preimmune serum (Fig. 2B). Control sections from healthy leaves did not show any specific gold labeling when incubated with preimmune or anti-P23 serum (data not presented).

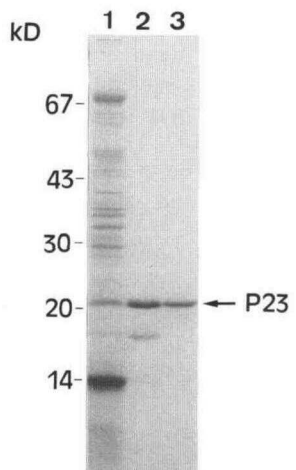

Figure 1. Purification of the viroid-induced tomato PR protein P23. Polypeptides present in each purification step were separated by SDS-PAGE. Lane 1, Analysis of crude acidic extracts from CEVd-infected tomato leaves. Lane 2, Proteins present after SP-Sephadex chromatography. Lane 3, Final preparation of P23 after Mono S fast protein liquid chromatography. Molecular mass markers are indicated on the left. The arrow on the right indicates position of the P23 protein.

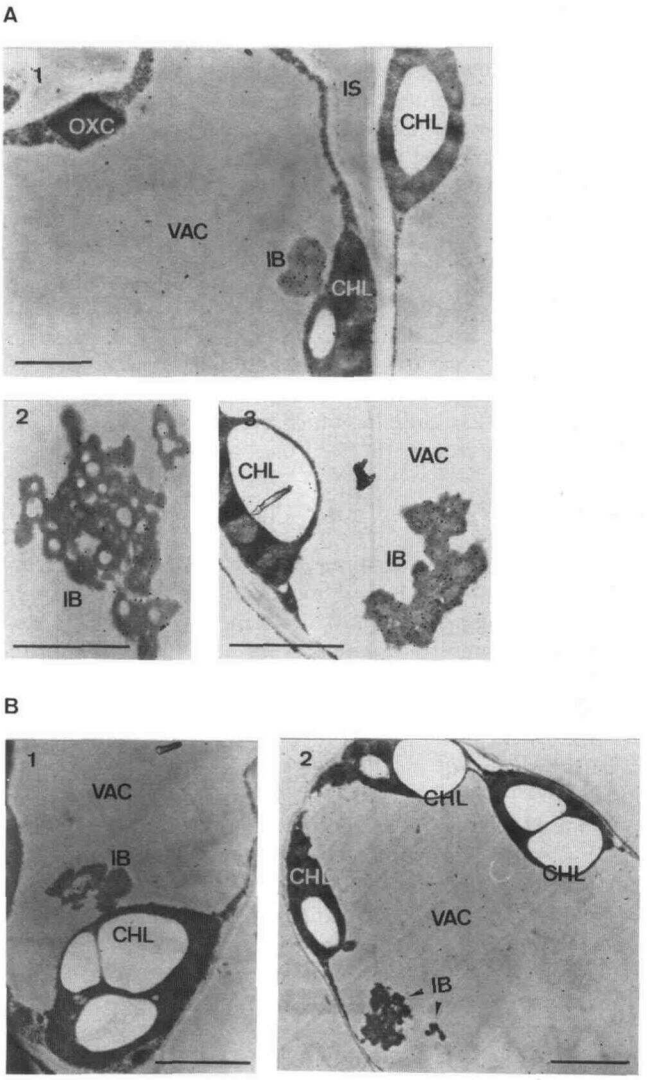

Figure 2. Immunocytochemical localization of P23 protein in ultrathin sections of CEVd-infected tomato leaf tissue. A, P23 antisera were used to reveal the in situ subcellular localization of P23. 1, General view of part of a leaf mesophyll cell in which the selective binding of an inclusion body can be observed. 2 and 3, Different shapes displayed by vacuolar inclusion bodies from CEVd-infected leaf tissue showing a high density of labeling. B, Ultrathin sections of CEVd-infected leaf tissue were incubated with rabbit preimmune serum as a control. 1 and 2, Different views of mesophyll cells containing vacuolar inclusion bodies. Only background levels, but not specific labeling, can be observed after treatment with protein A-colloidal gold. VAC, Vacuole; CHL, chloroplast; IB, inclusion body; IS, intercellular space; OXC, oxalate crystal. Bars correspond to 1 μm.

Antifungal Activity of the Viroid-Induced Tomato PR P23 Protein

The amino-terminal sequence of the recently described *Phytophthora*-induced antifungal tomato AP24 protein (Woloshuk et al., 1991) matches perfectly our previously published sequence of the viroid-induced tomato PR P23 (Rodrigo et al., 1991). There are also a number of other reports

that thaumatin-like proteins are antifungal (Vigers et al., 1991, 1992). When P23 was tested for antifungal activity, this viroid-induced PR protein inhibited the growth of a number of phytopathogenic fungi, although with different inhibitory activities. Increasing concentrations up to 50 μg mL^{-1} resulted in more than 80% inhibition of the growth of *T. roseum* and about 75% inhibition of *F. oxysporum* f. sp. *lycopersici* (Table I). P23 also diminished significantly the growth rate of *P. citrophthora*, although its effect against this fungus was less outstanding. P23 showed little inhibitory effect against *C. coccodes*. The antifungal activity of P23 was practically lost after boiling (see last row of Table I). The residual activity present after denaturation might be explained by the large number of Cys residues (16) present in this thaumatin-like protein, which are involved in eight disulfide bonds in thaumatin (De Vos et al., 1985).

Sequence Analysis of P23 cDNA Clones

Twelve positive clones were isolated from viroid-induced and ethylene-induced cDNA libraries. All 12 clones, although of different lengths, contained the same sequence. Figure 3 shows the complete sequence of the viroid-induced pTCP23.1 clone, which corresponds to the largest insert. We used the previously reported peptide sequence of viroid-induced tomato PR P23 (Rodrigo et al., 1991) to correctly frame translation of the pTCP23.1 insert. The sequence includes an open reading frame of 699 nucleotides, coding for a peptide of 233 amino acids. Although no ATG codon was found, the first eight amino acids should correspond to the signal peptide, as deduced from the previously reported N-terminal sequence of the mature P23 (Rodrigo et al., 1991). Following the TAA stop codon, there is an untranslated AT-rich region containing two consensus AATAAA polyadenylation boxes. The poly(A) tail present at the end of pTCP23.1 has not been included, because we have found even larger untranslated regions beyond that point in other P23-coding clones (e.g. up to 42 more nucleotides in the ethylene-induced pTEP23.3 clone).

Sequence comparison between the cDNAs corresponding to the viroid-induced P23 protein (pTCP23.1) and the salt stress-induced NP24 protein (pNP24, EMBL accession No. M21346) indicates 89% similarity between these two clones,

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1    GCTTTTGTGACTTACACTTATGCTGCCACTTTCGAGGTACGCAACAACTGTCCATACACC
1    A F V T Y T Y A A T F E V R N N C P Y T

61   GTCTGGGCGGCGTCGACCCCAATAGGCGGTGGTCGACGTCTTGATCGAGGCCAAACATGG
21   V W A A S T P I G G G R R L D R G Q T W

121  GTCATCAATGCACCGAGGGGCACTAAGATGGCACGTATATGGGGTCGTACGAATTGCAAC
41   V I N A P R G T K M A R I W G R T N C N

181  TTTGATGGTGCTGGTAGAGGTTCATGTCAGACTGGTGATTGTGGTGGGGTCTTGCAATGT
61   F D G A G R G S C Q T G D C G G V L Q C

241  ACCGGGTGGGGCAAACCACCAAACACCCTGGCCGAGTACGCCTTGGACCAGTTTAGCAAC
81   T G W G K P P N T L A E Y A L D Q F S N

301  CTAGATTTCTGGGACATTTCTTTAGTCGATGGATTTAATATTCCAATGACTTTCGCCCCG
101  L D F W D I S L V D G F N I P M T F A P

361  ACCAATCCTAGTGGAGGGAAATGCCATGCAATTCATTGTACGGCTAATATAAATGGTGAA
121  T N P S G G K C H A I H C T A N I N G E

421  TGTCCTGGTTCACTTAGGGTACCCGGAGGATGTAACAATCCTTGTACCACGTTCGGAGGA
141  C P G S L R V P G G C N N P C T T F G G

481  CAACAATATTGTTGCACACAAGGTCCATGTGGCCCTACTGATTTGTCGAGATTTTTCAAA
161  Q Q Y C C T Q G P C G P T D L S R F F K

541  CAAAGATGTCCTGATGCGTATAGCTACCCACAAGATGATCCTACTAGCACATTTACTTGC
181  Q R C P D A Y S Y P Q D D P T S T F T C

601  CCTAGTGGTAGTACAAATTATAGGGTTGTTTTTTGTCCTAATGGTGTTACTAGCCCAAAT
201  P S G S T N Y R V V F C P N G V T S P N

661  TTCCCCTTGGAGATGCCCTCAAGTGATGAAGAGGCTAAGTAAAATTGAGTCACTTTCTTT
221  F P L E M P S S D E E A K *

721  TAAATTGCTTGAAGTAGTCGAGTTATATAATTGGCTTGTAATAAACCTAATATAATTACA

781  TGAATAAAAGTCACATCATCACAAATATGTTGTTTCGAATATTATTTTATGTATATTTTG

841  TTATT
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Figure 3. Nucleotide sequence and deduced amino acid translation of pTCP23.1 insert. Peptide sequences in boldface correspond to the previously reported P23 amino acid sequence (Rodrigo et al., 1991). Consensus polyadenylation boxes are underlined.

Table I. *Effect of tomato PR P23 on growth of different plant pathogen fungi*

The numbers indicate the percentage of surviving colonies of *T. roseum*, *F. oxysporum*, and *C. coccodes* and the percentage of radial growth of *P. citrophthora* in the presence of the indicated amounts of P23, with respect to controls lacking this protein. As an additional control, samples containing the highest concentration of P23 were boiled for 5 min before being assayed with the fungi.

Fungus	P23 Concentration (μg mL^{-1})				
	0	5	25	50	50 (boiled)
T. roseum	100	74	51	17	80
P. citrophthora	100	87	65	41	85
F. oxysporum f. sp. *lycopersici*	100	79	58	24	78
C. coccodes	100	99	87	79	100

which corresponds to a 6% discrepancy in peptide sequence. However, only a 40% similarity and some gaps are found in the noncoding region. On the other hand, the P23 clone was identical with a recently reported tomato clone (TPM1, EMBL accession No. X66416) induced by the tomato planta macho viroid (Ruiz-Medrano et al., 1992) but had a single difference in the coding region (nucleotide position 191, C instead of A) and the corresponding amino acid sequence (Ala instead of Asp). Additionally, the pTCP23.1 insert contained a single-nucleotide gap at the untranslated region, located between nucleotides 740 and 741, as compared to TMP1. It is also pertinent to note that the poly(A) tail of the pTCP23.1 insert extends 40 nucleotides further than reported by Ruiz-Medrano et al. (1992) for the TPM1 clone.

Induction of PR P23 mRNA

Viroid infection involves the accumulation of a number of PR proteins in tomato plants, including P23. The presence of this protein in crude leaf extracts from CEVd-infected plants was selectively examined by immunoblotting using P23-specific antisera (Fig. 4). No trace of P23 protein was detected in crude leaf extracts from healthy plants. Consistent with this observation, northern blot analysis detected the P23 mRNA (approximately 1 kb) in viroid-infected plants but not in healthy ones.

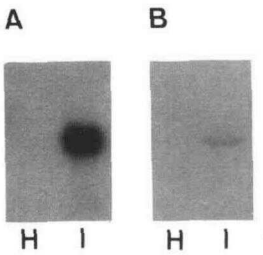

Figure 4. Induction of P23 message and protein by CEVd infection. A, Northern analysis of total RNA (15 μg) from healthy tomato leaves (H) and CEVd-infected tomato leaves (I), probed with ^{32}P-labeled pTCP23.1 cDNA. B, Western blot analysis using anti-P23 antisera of crude leaf extracts (50 μg protein) from healthy (H) and CEVd-infected (I) tomato leaves.

Ethylene has been described as a powerful inducer of PR proteins (Granell et al., 1987; Bol et al., 1990), and we have proposed that this plant hormone may be considered as a second messenger in viroid pathogenesis (Conejero et al., 1990). As shown in Figure 5, P23 accumulated in tomato leaf discs incubated in the presence of 1 mM 2-chloroethylphosphonic acid (ethephon, an ethylene-releasing agent). The amount of this protein in leaf tissue increased with ethylene incubation time. P23 mRNA was first detectable after 6 h of incubation with ethylene and progressively accumulated in the presence of this agent in parallel with the accumulation of the protein (Fig. 5).

P23 Gene Copy Number

To avoid cross-hybridization of tomato osmotin NP24 gene sequences with the pTCP23.1 probe because of their high level of homology in the coding region, we prepared a P23-specific probe, taking advantage of the low homology displayed by these two sequences after the translational stop

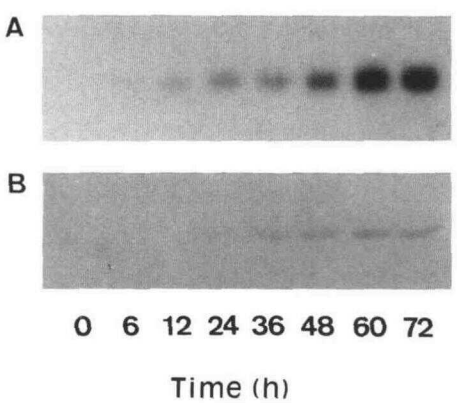

Figure 5. Ethylene induction of P23 mRNA and P23 protein. Tomato leaf discs were incubated in the presence of 1 mM ethephon for the indicated times. A, Total RNA was isolated from the different samples and analyzed by northern blot using ^{32}P-labeled pTCP23.1 cDNA as a probe. B, Crude protein extracts from the different sets of leaf discs were subjected to SDS-PAGE, electroblotted onto nitrocellulose membranes, and specifically revealed with P23 antisera as described in "Materials and Methods."

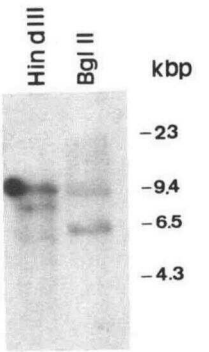

Figure 6. Southern analysis of genomic tomato DNA digested with *Hin*dIII (left) and *Bgl*II (right) and probed with the *Xho*I/*Hin*fIII fragment of pTCP23.3; washing was at high stringency. DNA molecular size markers are indicated on the right in kb (kbp).

codon. An *Hin*fI/*Xho*I digest of the pTEP23.3 clone yielded a 182-nucleotide AT-rich untranslated sequence showing only a 40% homology to the NP24 counterpart. The P23-specific probe identified up to three genomic sequences derived from restriction of tomato DNA with *Hin*dIII and *Bgl*II (Fig. 6).

DISCUSSION

The recently reported in vitro antifungal activity of osmotin-like proteins (Woloshuk et al., 1991) and other thaumatin-like proteins (Vigers et al., 1991, 1992) shed new light on our understanding of the biological role of this protein family. As we showed previously (Rodrigo et al., 1991), viroid infection of tomato plants results in the accumulation of a thaumatin-like PR protein (P23), which we identified as the salt stress-induced tomato osmotin NP24 because of the almost identical peptide sequence displayed by these two proteins. Further characterization presented in this work has revealed that tomato PR P23 is a vacuolar protein that seems to be selectively localized in association with inclusion bodies. The nature of these vacuolar structures remains obscure, but we have found them to be present in cells from viroid-infected tissues and absent from healthy ones (Vera et al., 1989a, 1989b). Additionally, tobacco cell cultures subjected to osmotic stress also contain inclusion bodies in which osmotin preferentially accumulates (Singh et al., 1987). Unlike the tomato PR proteins P1(p14) and P69, which are present in both vacuolar inclusion bodies and intercellular spaces (Vera et al., 1988, 1989a, 1989b), P23 was not detected in the apoplast. The specific vacuolar compartmentation of P23 is otherwise coincident with what is general for most basic PR proteins described to date (Bol et al., 1990).

The thaumatin-like nature of tomato P23 and the obvious peptide sequence identity with the *Phytophthora*-induced antifungal protein AP24 (Woloshuk et al., 1991) strongly suggested a biological role for P23 as a pathogen-induced antifungal factor. In this respect, we have been able to show that the tomato PR P23 protein also displays antifungal activity. This protein had no chitinase or β-1,3-glucanase activity (not shown) and inhibited the growth of *P. citrophthora*, a fungus

belonging to the Oomycetes, which have been described as insensitive to these hydrolytic enzymes (Mauch et al., 1988). The phytopathogenic fungi studied in this work showed different susceptibilities when assayed with equal amounts of P23, ranging from considerable inhibition to almost complete insensitivity. This differential sensitivity indicates that, as suggested before (Vigers et al., 1992), the particular features of the different fungi (e.g. cell wall components or membrane receptors) condition their differential susceptibility to P23. This protein may act against fungi by a mechanism similar to that described for the homologous permatins (Vigers et al., 1991). In this context, the data concerning the vacuolar presence of P23 suggest an antifungal function that is in synergy with vacuolar chitinases and β-1,3-glucanases by complementing their antifungal mechanisms.

P23 cDNA cloning provided important complementary information regarding the cellular and molecular biology of this protein. The most outstanding fact was the difference in nucleotide sequence with the salt stress-induced tomato osmotin. Before availability of the cDNA sequence of P23, we identified this protein as the salt-induced tomato NP24 (Rodrigo et al., 1991) and suggested that viroid infection could also involve the triggering of osmotins. However, the present data indicate that these two proteins are encoded by different genes, because they display an 11% divergence in the coding region and only a scattered matching of less than 40% homology in the noncoding, untranslated sequences. This also suggests that P23 and NP24 genes, although encoding for similar proteins, might follow different patterns of expression and regulation. No cDNA resembling NP24 was found in either viroid- or ethylene-induced tomato cDNA library, which were prepared from leaf tissue. Additionally, all of the P23 clones isolated were 100% homologous to the sequence we are presenting in this work, irrespective of their induction by ethylene or CEVd.

The first 5′-nucleotide sequence of pTCP23.1 encodes a peptide sequence that is upstream from the N terminus found on the mature protein (Rodrigo et al., 1991). This probably corresponds to the peptide signal sequence responsible for protein sorting (see Chrispeels, 1991, for review). Additionally, protein targeting to the plant vacuole depends on the presence of C-terminal propeptides (Bednarek et al., 1990; Neuhaus et al., 1991). As has been shown recently (Melchers et al., 1992), the P23 homolog tobacco AP24 protein requires the removal of a C-terminal peptide to sort correctly intracellularly; otherwise, it is directed to the apoplast. In this respect, we have observed that P23 accumulates in the vacuole and is practically absent from the intercellular space. Because the deduced peptide sequence of P23 displays an AP24-homologous stretch beyond the last sequence that we had obtained from the mature protein (Fig. 3), it is also very likely that P23 undergoes such C-terminal posttranslational processing.

A major point is the induction of closely similar proteins by salt stress (King et al., 1988) and pathogens (Rodrigo et al., 1991; Woloshuk et al., 1991; Ruiz-Medrano et al., 1992). Despite their different inducibility and tissue specificity, the strong homologies between them imply that these proteins might have similar biological functions, including antifungal activity, although a role in osmotic adaptation cannot yet be excluded. In this respect, Kononowicz et al. (1992) proposed that osmotin could have a primary role in low water potential situations by protecting plasma membranes as a part of a mechanism causing permeabilization. These authors proposed that a secondary step would involve the use of this protein as a vacuole-associated permeabilizing agent acting against the plasma membrane of pathogens. Our data concerning the vacuolar presence of the antifungal, viroid-induced P23 protein support this idea.

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